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Genotyping of *Mycoplasma pneumoniae* isolates using real-time PCR and high-resolution melt analysis

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Abstract

Mycoplasma pneumoniae is an important respiratory pathogen, accounting for up to 25% of community-acquired pneumonia, and is a common cause of hospitalized pneumonia in otherwise healthy adults and children. *Mycoplasma pneumoniae* isolates can be classified into two main genomic groups (type 1 and type 2) based on sequence variation within the gene encoding the major adhesion molecule P1. Although numerous publications have described real-time PCR assays for the detection of *M. pneumoniae*, none has been able to discriminate the two genomic types. Here, a real-time PCR assay that can distinguish each type of *M. pneumoniae* utilizing high-resolution melt-curve analysis is reported. Using this method, 102 isolates obtained from patients from 1965 to the present, including those from recent outbreaks, were typed along with reference strains M129 (type 1) and FH (type 2). The results show that 55 isolates (54%) can be classified as type 1 and 47 isolates (46%) as type 2, and 100% correlation was demonstrated when compared with a standard PCR-restriction fragment length polymorphism typing procedure. Typing of isolates obtained from recent outbreaks in the USA has revealed the presence of both types. This assay provides a rapid, reliable and convenient method for typing *M. pneumoniae* isolates and may be useful for surveillance purposes and epidemiological investigations, and may provide insight into the biology of *M. pneumoniae* distribution within populations.

Keywords: Genomic typing, genotyping, high-resolution melt, *Mycoplasma pneumoniae*, real-time PCR, subtyping
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Introduction

Mycoplasma pneumoniae is a significant cause of respiratory disease and community-acquired pneumonia, accounting for as high as 33% of hospitalized cases [1,2]. This frequent cause of community outbreaks usually presents as a mild, self-limiting respiratory illness, although it has been rarely associated with extrapulmonary complications, including neurological, cardiac and autoimmune manifestations [1,3–5]. Due to its minimal and highly uniform genome, genetic diversity among isolates of *M. pneumoniae* is sparse [6]. Most isolates can be categorized into one of two genomic groups (type 1 or type 2) based on sequence variation of the *P1* (MPN141) gene [6–8]. The P1 protein is an important immunogen and virulence factor, which forms heterogeneous complexes with other proteins and localizes at the attachment organelle to mediate adherence of *M. pneumoniae* to respiratory epithelial cells [9].

Currently, the standard typing method for distinguishing *M. pneumoniae* isolates involves PCR-restriction fragment length polymorphism (PCR-RFLP), which can be laborious and time-consuming [10–13]. Recently, Dumke *et al.* [14] described a nested-PCR followed by sequencing that can be used directly with clinical specimen extracts to distinguish each type and all known variants of *M. pneumoniae.* This assay, however, requires several rounds of PCR, gel purification, and sequencing analysis and thus is not ideal for generating rapid results. Real-time PCR followed by high-resolution melt (HRM) analysis has been used by several groups to discriminate genotypes or detect single nucleotide polymorphisms (SNPs) and, more recently, to provide greater genetic characterization of microbes [15,16]. This technique has also been applied to detect macrolide-resistant isolates of *M. pneumoniae* and to classify strains of *M. synoviae* into ten profiles, replacing PCR-RFLP as the standard typing mechanism [17,18].

In the present study, we targeted type-specific sequences within the *P1* gene to design a real-time PCR followed by

HRM analysis to discriminate between the two types of *M. pneumoniae*. An approximately 1900-bp fragment of the *P1* gene was amplified and characterized by HRM analysis to reliably separate type 1 and type 2 isolates. Using this assay, we screened 102 isolates of *M. pneumoniae* acquired by our laboratory since 1965, which are comprised of clinical strains from individual cases, surveillance studies, and outbreaks within the USA and Europe, including isolates from four recent outbreaks in the USA. We then compared the typing results with PCR-RFLP analysis.

Materials and Methods

Strains and isolates

Mycoplasma pneumoniae strains M129 (ATCC 29342) and FH (ATCC 15531) were used as the reference strains for type 1 and type 2, respectively. All *M. pneumoniae* isolates used in this study were isolated and grown in SP4 media as previously described [19].

DNA isolation

DNA was extracted from liquid cultures using either the QIAmp DNA mini kit (Qiagen, Germantown, MA, USA), or the Gentra Puregene Blood kit (Minneapolis, MN, USA), according to the manufacturer's instructions.

Real-Time PCR HRM Assay

Primers Mpt-F (5′-TTAGCAGCTCTTCCCGACAA-3′) and Mpt-R (5′-ACATCGTCATTCATCTTTGCGGC-3′) were designed to amplify an approximately 1900-bp target region variable between types 1 and 2 of *M. pneumoniae,* partially spanning the RepMP2/3 (repetitive) region within the *P1* gene. Real-time PCR assays were performed on the Corbett Rotor-Gene 6000 real-time rotary analyser (Sydney, Australia) using the Universal SYBRGreenER qPCR kit (Invitrogen, Carlsbad, CA, USA). Each tube contained the following per reaction: 12.5 μL of 2× mix, a final concentration of 250 nM of each primer above, 5 μL of template at 1 ng/μL, 1 μL 10 mM dNTP nucleotide mix (Promega, Madison, WI, USA), and 0.25 μL platinum *Taq* polymerase (5 U/μL) (Invitrogen) and Nuclease-Free Water (Promega) to a total volume of 25 μL. All samples were tested in triplicate. Amplification conditions were as follows: 95°C for 2 min, followed by 35 cycles of: 95°C for 30 s, 55°C for 15 s, and 68°C for 90 s. The HRM was performed between 84°C and 90°C. Data were collected at 0.05°C/s intervals. HRM data were normalized by setting the pre-melt baseline regions between 85.25°C and 85.5°C, and the post-melt baseline regions between 88.25°C and 88.5°C.

RFLP

Previously published primer sets ADH1/ADH2 and ADH3/ADH4 were used to amplify an approximately 2300-bp and an approximately 2500-bp fragment, respectively, of the *P1* gene [10]. Briefly, each reaction contained 12.5 μL 2× Platinum Quantitative PCR SuperMix-UNG (Invitrogen), a final concentration of 1 μM each of primers ADH1 and ADH2, or AHD3 and ADH4, 0.25 μL platinum *Taq* polymerase (5 U/μL), 1 μL 10 mM dNTP nucleotide mix, 100 ng of template, and nuclease-free water to a volume of 50 μL. Amplification was performed on the Stratagene Mx3005 instrument (La Jolla, CA, USA). Reaction conditions were as follows: 95°C for 2 min, followed by 40 cycles of: 94°C for 30 s, 55°C for 30 s, and 68°C for 3 min [13]. The PCR products were separately subjected to *Hae*III (NEB, Inc., Ispwich, MA, USA) digestion as described previously [10].

Sequencing and Analysis

The *c.*1900 bp amplicon in the real-time PCR-HRM assay was sequenced following gel purification of the amplified products using the GENECLEAN TurboKit (MP Biochemicals, Irvine, CA, USA). Sequencing reactions were performed with the Big Dye Terminator v3.1 kit (Applied Biosystems Inc., Foster City, CA, USA) with approximately 40 ng DNA and a final concentration of 3.3 μM of each primer in a 20 μL reaction. Sequencing reaction conditions were as follows: 96°C for 1 min, followed by 30 cycles of 96°C for 10 s, 55°C for 10 s, and 60°C for 4 min. The sequence reaction products were cleaned using CentriSep 8 spin columns (Princeton Separations, Adelphia, NJ, USA), and analysed on a 3130xl ABI Prism Genetic Analyzer (Applied Biosystems Inc.).

Results

Real-time PCR and HRM subtyping

Fig. 1 shows the distinct typing results of the real-time PCR-HRM assay on a representative number of strains and isolates. In the normalized graph mode (Fig. 1a), the initial fluorescence signal at the pre-melt stage is set to 100%,

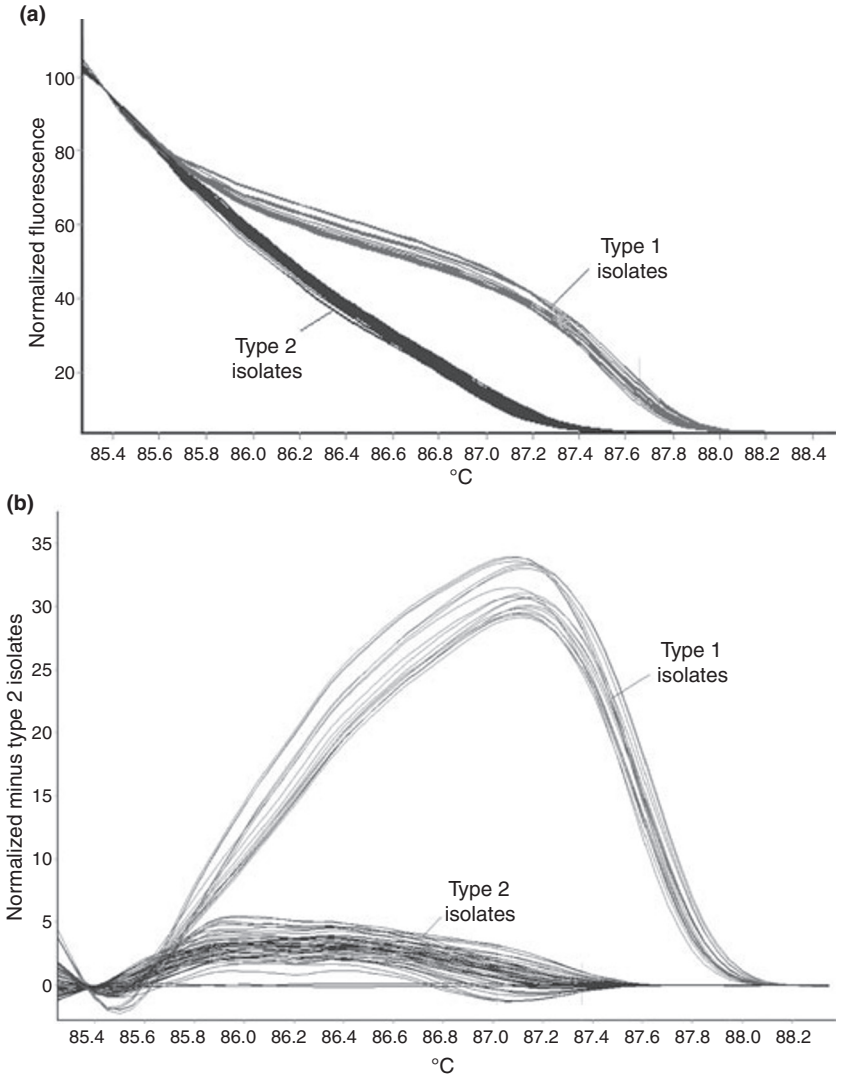

FIG. 1. Real-time PCR high-resolution melt (HRM) assay. (a) The HRM profile for type 1 and type 2 isolates in the normalized graph mode; and (b) the difference graph illustrates deviations between types 1 and 2. The type 2 isolates have been selected as the standard, displaying the deviations of type 1 isolates from the type 2 isolates.

whereas the final fluorescence level at the post-melt stage is set to 0%, allowing for the type-specific melt profiles generated by the amplicons of each type to be viewed. The amplicon of type 2 isolates consistently shows a lower melt profile than that of type 1 isolates, which displays a significant shift of the profile to the right. Fig. 1b displays the data using a difference graph, in which type 2 isolates have been selected as the standard from which to show deviations. This alternate viewing of the same data illustrates the deviations between the types. The 102 isolates all reliably sorted into one of two distinct groups: type 1 (54%) or type 2 (46%). These typed 102 isolates included those acquired by the CDC during four outbreaks which occurred in the USA from 1999 to 2007. Isolates of both *M. pneumoniae* types were obtained during this period and show a type 2 homogeneity in two outbreaks: Indiana, 1999 (5/5) and New Hampshire, 2007 (2/2). A type 1 homogeneity was observed in the other two outbreaks: Maine, 2007 (4/4) and Rhode Island, 2006–2007, with the exception of a single type 2 isolate among 11 type 1 isolates (10/11).

Sequence analysis

Sequencing of the *c.*1900-bp PCR products for reference strains M129 (type 1) and FH (type 2), along with a clinical isolate of each type, 2p (type 1) and 1136 (type 2), was performed in order to compare sequence differences responsible for the type-specific melting profiles. Sequence alignment of the variable regions between the types is shown in Fig. 2. The sequence results comparing M129 strain and isolate 2p are identical, whereas two nucleotide mismatches are observed between FH strain and isolate 1136. Significant differences are seen between type 1 and type 2 reference strains and isolates, including several gaps/insertions.

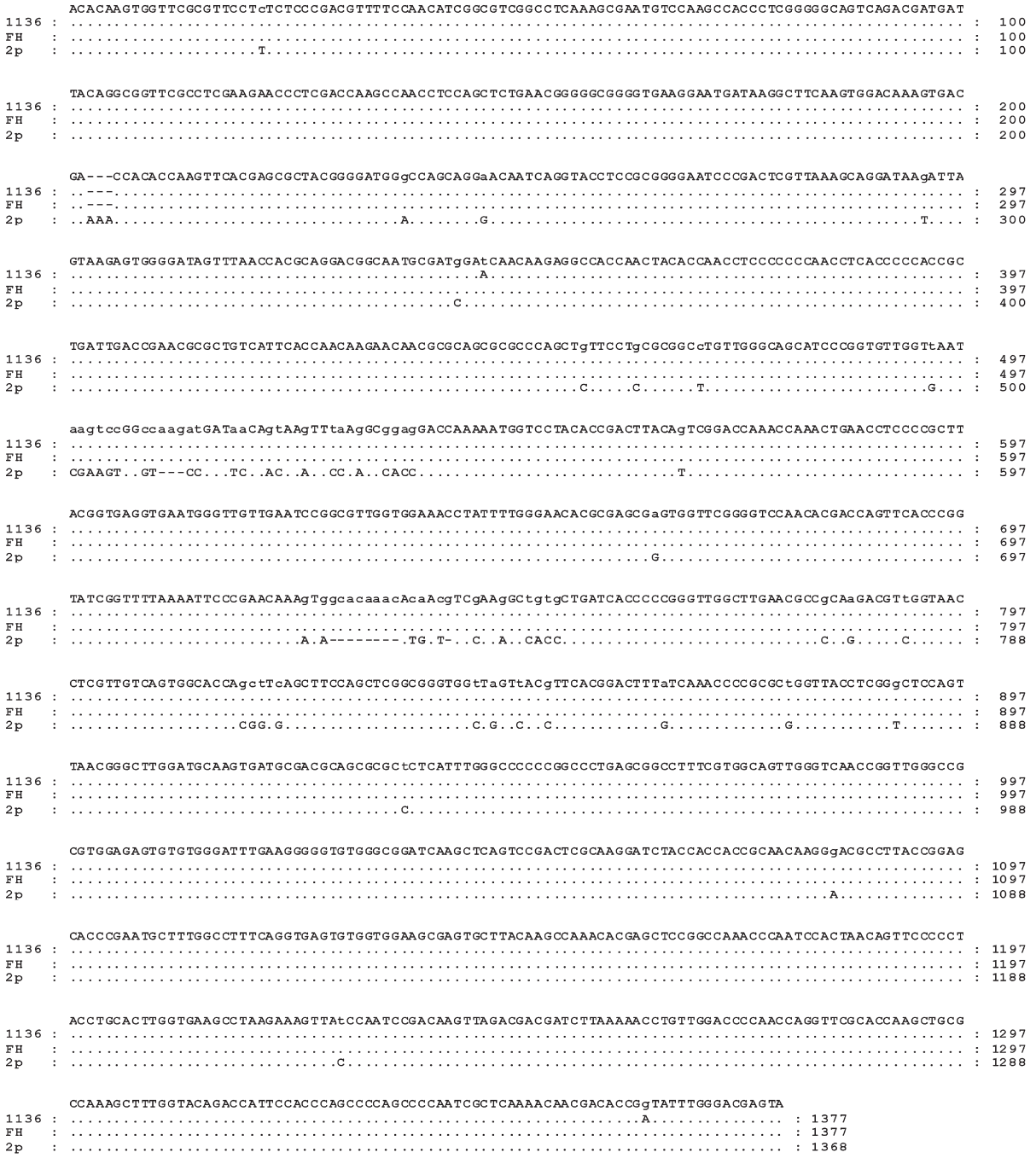

FIG. 2. Sequence alignment. Alignment of the variable regions derived from the *c.*1900-bp product of *Mycoplasma pneumoniae* clinical isolates 2p (type 1) and 1136 (type 2) and the prototypical type 2 reference strain FH. The type 1 reference strain M129 sequence [24] is identical to 2p. The top row illustrates the consensus sequence. The accession numbers for the sequences are FJ215694, FJ215693, and FJ215695 for strains 1136, FH, and 2p, respectively.

RFLP analysis

To confirm and validate this PCR-HRM typing assay, we performed RFLP analysis of the ADH1/ADH2- and ADH3/ADH4-generated fragments of P1, digested by *HaeIII* on all 102 isolates. PCR-RFLP typing results correlated 100% with the real-time PCR followed by HRM. Fig. 3a,b shows the banding patterns of a representative number of isolates of each type after enzyme digestion.

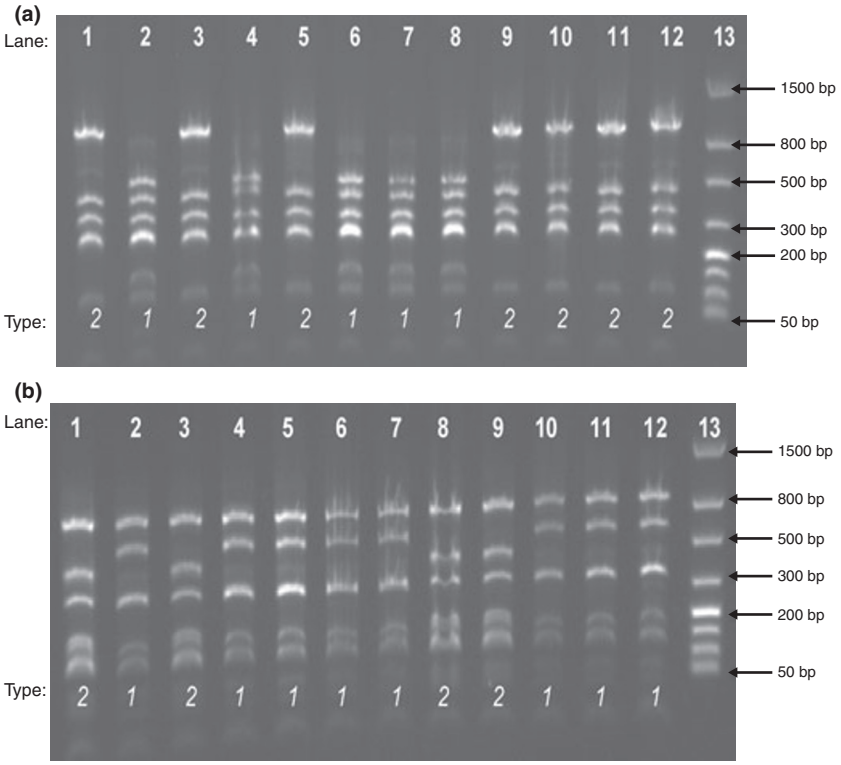

FIG. 3. Restriction fragment length polymorphism analysis of *Hae*III-digested PCR fragments. M129 and FH were used as the reference strains for type 1 and type 2, respectively. Characteristic banding patterns for each type are shown. A broad-range molecular weight marker is shown in lane 13 for both a and b. (a) Representative gel illustrating *Hae*III-digested type-specific banding patterns from fragment 1 (generated by primers ADH1/ADH2) for ten isolates along with reference strains. Type 1 isolates (lane 2 – M129; lanes 4, 6, 7, 8) and type 2 isolates (lane 1 – FH; lanes 3, 5, 9, 10, 11, 12) are shown; and (b) representative gel illustrating *Hae*III-digested type-specific banding patterns from fragment 2 (generated by primers ADH3/ADH4) for ten isolates along with reference strains. Type 1 isolates (lane 2 – M129; lanes 4, 5, 6, 7, 10, 11, 12) and type 2 isolates (lane 1 – FH; lanes 3, 8, 9) are shown.

Discussion

We targeted a c.1900-bp variable region within the *P1* gene of *M. pneumoniae* to design a PCR-HRM assay capable of genotyping type 1 and type 2 isolates of this agent. Although PCR-HRM assays typically target smaller regions (<500 bp) to discriminate minor changes [20], including SNPs, this study reports an alternative use of this technology. Complex melting profiles have been reported to provide a better means of distinguishing differences than simple, single-peaked melting profiles that are found within smaller amplicons [21]. The variable region of the *P1* gene (MPN141) of *M. pneumoniae* spans a greater area and thus provides an ideal target for designing a reliable discriminatory assay for typing this respiratory pathogen.

In this study, the typing results of the 102 isolates obtained since 1965 showed a virtually equivalent distribution (54% type 1, and 46% type 2). A remarkably clear separation of type 1 and type 2 isolates can be seen in both the 'normalized' and 'difference' graph modes (Fig. 1), with some minor intratype variability. This separation is based on the melting dynamics of type-specific sequence domains within the targeted region as shown in Fig. 2. These data display 100% concordance with the current reference standard typing method of PCR-RFLP, which we performed using each isolate to validate our assay (Fig. 3). Further analysis of the sequence data within Fig. 2 reveals numerous base substitutions and gaps within the region that account for the unique melting profiles of type 1 vs. type 2 isolates. Although very slight intratype melt variations corresponding to the two nucleotide mismatches between FH strain and isolate 1136 were noted (data not shown), major differences, as shown between type 1 and type 2, were not observed.

Methods for differentiating isolates may serve numerous purposes. For instance, monitoring epidemiological trends of *M. pneumoniae* outbreaks may lead to greater understanding

of the biology and communicable nature of this agent and yield insight into its ability to cause cyclic epidemics. A recent retrospective genotyping analysis of isolates collected over a 10-year period by Kenri *et al.* [11] provides evidence of the phenomenon of type-switching occurring approximately every 7 years in Japan. Sasaki *et al.* [10] had previously documented distinct shifts in *M. pneumoniae* types over a 20-year period in Japan, with intervals of 4 years between epidemics; however, they were unable to correlate this shift definitively with epidemic cycles. Type-switching has been proposed to occur due to population-based immune pressure and may explain the cyclic nature of both type-specific outbreaks and subsequent immunity [22]. Our data from four recent outbreaks clustered in the USA indicate that both types are currently circulating within the population and, in the case of the Rhode Island 2006–2007 outbreak, are concurrently present within a single outbreak. Collectively, *M. pneumoniae* typing studies have illustrated the difficulty in clearly delineating the correlation of type switching with immunity, epidemics and prevalence in the population. Large-scale surveillance and characterization of the *M. pneumoniae* types may improve this understanding.

Additionally, retrospective and prospective typing analysis of *M. pneumoniae* isolates may also allow researchers to study possible relationships among genotypes and their potential role in virulence, infectivity rates, host preferences (age, sex, race, etc.) and immune pressures that may lead to type-switching. Thus far, no clear trends in subtype-specific virulence or infectivity have been demonstrated [23]. The procedure could facilitate a more rapid and high-throughput ability to type isolates, expediting more thorough and comprehensive studies. Lastly, because the P1 antigen is known to be immunodominant and is a major basis for characterizing *M. pneumoniae* types, relevant typing information may aid in future vaccine development.

This is the first report of a rapid and simple real-time PCR method that is able to discriminate reliably between the two dominant types of *M. pneumoniae*. This approach provides a marked improvement over the more laborious procedures of PCR-RFLP, pulsed-field gel electrophoresis, and nested PCR followed by sequencing and less reliable randomly amplified polymorphic DNA tests that are currently used. Furthermore, this methodology may also allow for greater characterization of isolates, including the identification of variants of each type. Research studies are ongoing, with the aim of expanding the applications of this assay to include: (i) testing of clinical specimens; (ii) implementing for surveillance purposes; (iii) prospective use during outbreaks; and (iv) possible use to monitor the genetic evolution of this important virulence gene.

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Transparency Declaration

This work was supported in full by the government of the USA. The authors declare no dual or conflicting interests.

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